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1 Engineering mannitol biosynthesis in *Escherichia coli*
2 and *Synechococcus* sp. PCC 7002 using a green algal
3 fusion protein

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10 **Abstract**

11 The genetic engineering of microbial cell factories is a sustainable alternative to the chemical
12 synthesis of organic compounds. Successful metabolic engineering often depends on
13 manipulating several enzymes, requiring multiple transformation steps and selection markers,
14 as well as protein assembly and efficient substrate channeling. Naturally occurring fusion
15 genes encoding two or more enzymatic functions may offer an opportunity to simplify the
16 engineering process and to generate ready-made protein modules, but their functionality in
17 heterologous systems remains to be tested. Here we show that heterologous expression of a
18 fusion enzyme from the marine alga *Micromonas pusilla*, comprising a mannitol-1-phosphate
19 dehydrogenase and a mannitol-1-phosphatase, leads to synthesis of mannitol by *Escherichia*
20 *coli* and by the cyanobacterium *Synechococcus* sp. PCC 7002. Neither of the heterologous
21 systems naturally produces this sugar alcohol, which is widely used in food, pharmaceutical,
22 medical and chemical industries. While the mannitol production rates obtained by single-gene
23 manipulation were lower than those previously achieved after pathway optimization with

24 multiple genes, our findings show that naturally occurring fusion proteins can offer simple
25 building blocks for the assembly and optimization of recombinant metabolic pathways.

26 **Keywords**

27 Synthetic biology, Fusion Protein, Mannitol, Cyanobacteria, *Micromonas*.

28

Microbial cell factories, particularly photosynthetic chassis that consume carbon dioxide as their sole carbon source, are an attractive alternative to chemical synthesis ¹. They present a sustainable approach to producing an array of substances with usages in food, pharmaceutical, nutraceutical and cosmetic industries.

Biological production platforms struggle to compete economically, particularly for the production of low value commodity chemicals and materials ². A recurring problem is performance, which is measured as titer, yield and productivity. Artificial metabolic pathways inherently generate imbalances in pathway flux resulting in (toxic) intermediate and side product accumulation, growth inhibition, and ultimately low product yield ³. Therefore, individual modifications (e.g. the introduction of a single enzyme or pathway) are not sufficient to achieve industrially relevant titers. As a result, cell factory development requires the introduction of several genes to synthesize the desired product, the overproduction of precursors, and the deletion of competing pathways ⁴⁻⁵.

The use of fusion genes, generated from previously separate genes, can help to simplify the biological engineering process ⁶. Firstly, reducing the number of genes required for a synthetic pathway simplifies the design and assembly of synthetic expression constructs as well as the transformation and selection of producing strains. Secondly, reducing the number of enzymes simplifies assembly of protein complexes encoded by separate genes and stoichiometric optimization to balance pathway flux. Furthermore, catalytic sites are brought in closer proximity, thus enhancing substrate channeling and reducing intermediate accumulation and/or loss to competing pathways ⁷. Additionally, transit times for intermediate products between catalytic sites are significantly reduced thus improving biosynthetic efficiency.

D-Mannitol is a sugar alcohol, or polyol, ubiquitous in prokaryotes and eukaryotes, except for Archaea and animals. It has desirable properties for food, pharmaceutical, medical and chemical industries ⁸. Based on a recent analysis of mannitol markets (<http://www.grandviewresearch.com/industry-analysis/mannitol-market>; last accessed on 01/11/2017), demand for this polyol, driven by an increasing use in the food and

57 pharmaceutical sectors, is expected to reach 35.9 million kg representing a market of USD
58 418.3 million by 2024.

59 Mannitol can be produced by extraction, chemical synthesis or biosynthesis. Traditionally,
60 mannitol was harvested from plant material which was seasonal and yields highly variable ⁹.
61 To stabilize mannitol supplies, commercial production now mostly occurs through chemical
62 hydrogenation of fructose-glucose syrups. However, poor selectivity of the nickel catalyst
63 results in a mixture of mannitol and sorbitol which is relatively difficult to separate and thus
64 costly ¹⁰. Chemical synthesis can be improved by altering the substrate, e.g. isomerizing
65 glucose to fructose by enzymatic conversion; however, enzyme availability and added costs
66 of additional steps prevent this from being economical.

67 The chemical industry is now looking to bio-based production methods to reduce costs and
68 environmental impact. The most successful approach to mannitol biosynthesis currently uses
69 heterofermentative lactic acid bacteria. Under anaerobic conditions, these organisms reduce
70 fructose using the native enzyme mannitol dehydrogenase. This approach requires an
71 external sugar supply that is a) predominantly obtained from traditional crops, e.g. corn and b)
72 a major cost for biosynthesis of commodities such as mannitol. External carbon sources and
73 associated costs can be eliminated from the bio-production pipeline by using photosynthetic
74 organisms, which assimilate atmospheric carbon dioxide into sugars via the Calvin cycle ¹¹.

75 Cyanobacteria represent an incredibly diverse phylum of phototrophic prokaryotes that are
76 being developed for photosynthetic bio-production ¹²⁻¹⁴. One particularly attractive chassis is
77 the unicellular euryhaline cyanobacterium *Synechococcus* sp. PCC 7002 due to its reported
78 fast growth and tolerance of high salt, light and temperature ¹⁵⁻¹⁷. Importantly, *Synechococcus*
79 is amenable to natural transformation ¹⁸⁻¹⁹ and its genome is fully sequenced
80 (<http://genome.microbedb.jp/cyanobase/SYNPCC7002>). Some molecular tools to engineer
81 *Synechococcus* have been developed including transformation vectors, promoters to control
82 transcription and ribosome binding sites to control translation ²⁰⁻²².

83 *Synechococcus* has previously been engineered to produce mannitol from fructose-6-
84 phosphate, an intermediate of the Calvin cycle ²³. This recombinant mannitol biosynthetic

pathway involved heterologous expression of two enzymes encoded by separate genes: (1) an *Escherichia coli* gene encoding mannitol-1-phosphate dehydrogenase (M1PDH), which catalyzes the reduction of fructose-6-phosphate (F6P) to mannitol-1-phosphate (M1P), and (2) an *Eimeria tenella* gene encoding phosphohistidine mannitol-1-phosphatase (M1Pase), which hydrolyses M1P to mannitol.

In recent years, new insights have been gained on the mannitol biosynthetic pathway, notably in algae. The first algal genes involved in mannitol production were identified in the model brown alga *Ectocarpus*²⁴, and biochemical characterization of the recombinant enzymes confirmed M1PDH and M1Pase function²⁵⁻²⁷. Analysis of the distribution and evolution of these mannitol biosynthetic genes across algal lineages showed that mannitol synthesis is more widely spread and diverse than initially thought²⁸. Interestingly, several fusion genes combining modules for M1PDH and M1Pase activities were identified, notably in marine green algae. For instance, Mipuc10g00620 (<http://bioinformatics.psb.ugent.be/orcae/annotation/Mipuc/current/Mipuc10g00620>) of *Micromonas pusilla* strain CCMP1545 is predicted to encode an enzyme expected to transform F6P directly into mannitol.

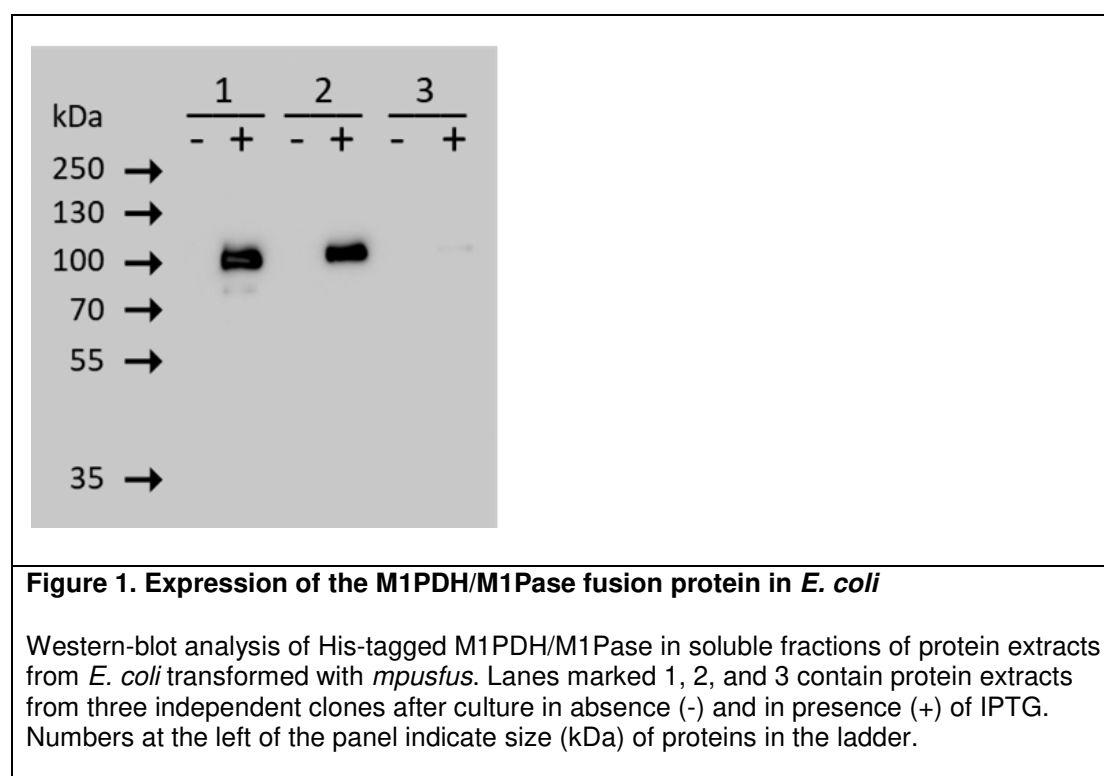
In this study, we explored whether the M1PDH/M1Pase fusion gene of *M. pusilla* strain CCMP1545 is functional when heterologously expressed in heterotrophic (*E. coli*) or photoautotrophic (*Synechococcus*) bacteria. To facilitate the engineering of *Synechococcus* we generated BioBrick-compatible molecular tools (vectors and ribosome binding sites). We found that both *E. coli* and *Synechococcus* produced mannitol when transformed with the fusion gene. The one-step mannitol production pathway provides an excellent starting point for further optimization of sustainable mannitol production in cyanobacteria.

Results

M1PDH/M1Pase is functional in E. coli

The M1PDH/M1Pase fusion gene of *Micromonas pusilla* strain CCMP1545 was codon optimized for expression in *Synechococcus* (see Methods) and we called the resulting

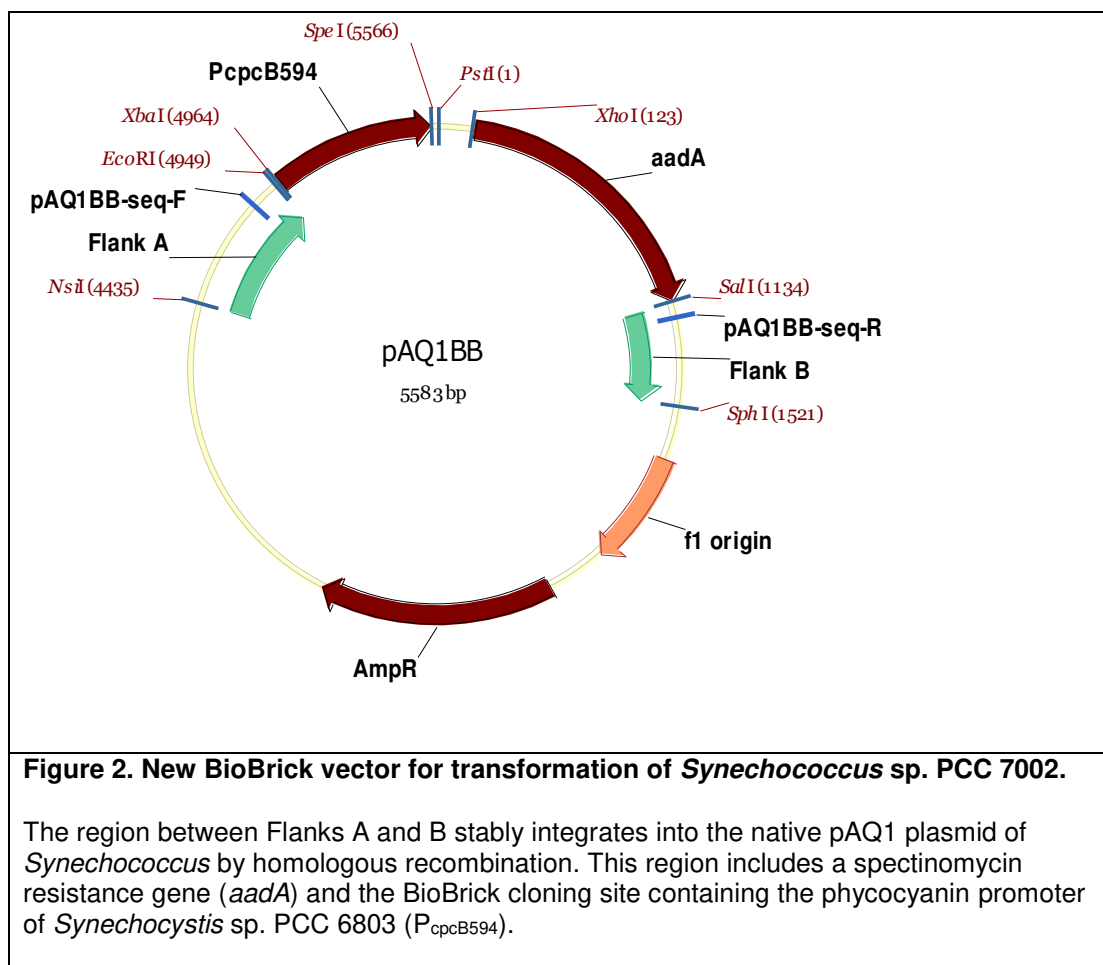
sequence *mpusfus*. To test expression and function in heterologous systems, *mpusfus* was first introduced into *E. coli* using the pFO4 vector that carries an IPTG-inducible expression system. A six-histidine tag was added to the 5' end of the gene. Western-blot of protein extracts from IPTG-induced cells revealed a band of the expected size while no band was detected in the controls without IPTG (Figure 1). The size of the primary band was close to the value of 94.8 kDa calculated for the full-length predicted amino acid sequence. This showed that a single fusion protein was produced from the fusion gene.



Mannitol was measured both in the culture media and in the cells after 20 hours of incubation in absence and in presence of IPTG. In 5 ml cultures with a final OD₆₀₀ of 2.5-3.0 we found 1 ± 0.05 mg of mannitol in the media and 0.08 ± 0.008 mg of mannitol inside the cells (n = 3 clones) after incubation with IPTG. Extrapolated to a 1-litre culture, total mannitol production was 218 ± 11.9 mg/L, of which 202 ± 10.7 mg (93 %) were exported into the medium and 16 ± 1.6 mg (7 %) were retained inside the cells. No mannitol was detected in cultures grown without IPTG. The results showed that the algal fusion gene produces a single protein in *E. coli* that catalyses the biosynthesis of mannitol.

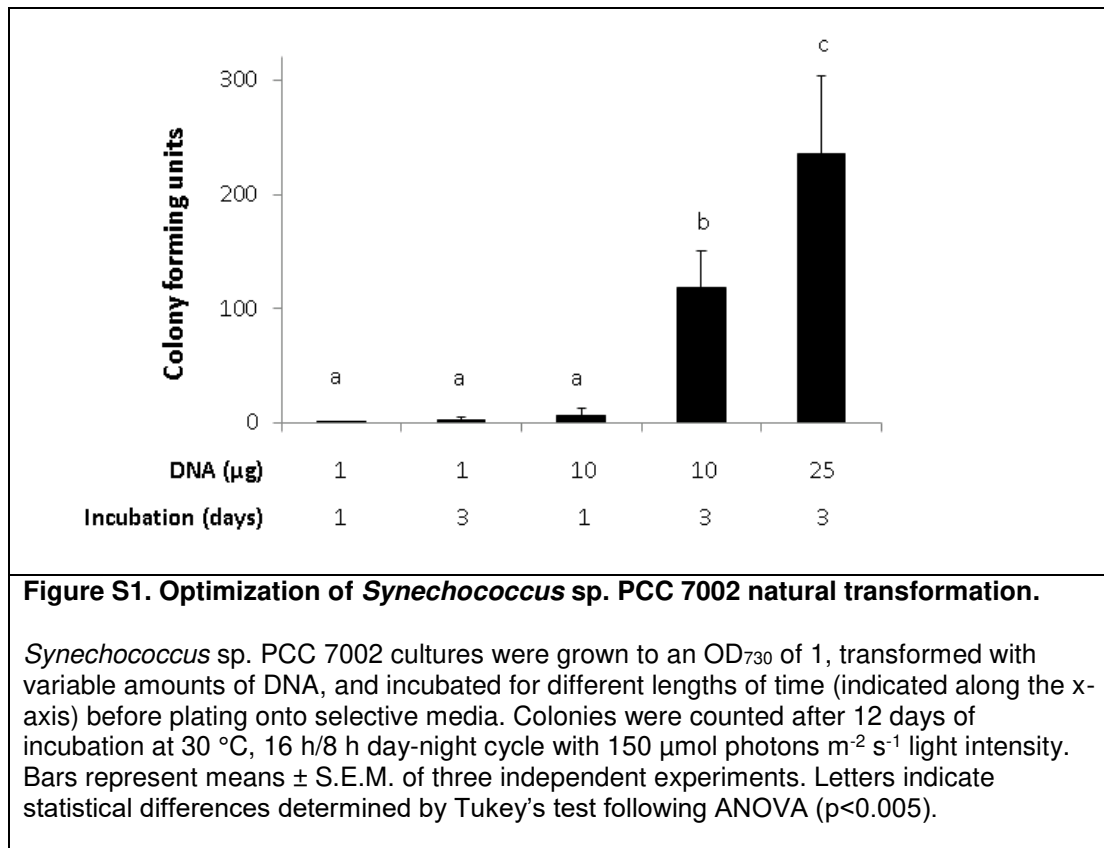
New molecular tools and protocols to engineer *Synechococcus* sp. PCC 7002

BioBricks represent the largest collection of standardized parts for genetic engineering ²⁹. While a number of genetic tools have been developed for *Synechococcus* ²⁰⁻²², none of them are compatible with BioBrick parts. In order to develop a BioBrick-compatible integration vector for *Synechococcus*, we reengineered an existing expression vector, pAQ1EX ³⁰. This vector targets the transgene to a neutral site in the native, high copy pAQ1 plasmid of *Synechococcus*. BioBrick prefix and suffix sequences containing the restriction enzyme recognition sites required for gene assembly were synthesized and inserted into the integrative region of the pAQ1EX vector. For ease of cloning, domestication of DNA parts is important whereby BioBrick restriction sites do not occur outside of the prefix and suffix sequences. An *Xba*I site occurring directly upstream of the selective *aadA* gene was therefore replaced with an *Xho*I site. Finally, the phycocyanin promoter of *Synechocystis* sp. PCC 6803, P_{cpCB594}, which has been reported to be a strong promoter in cyanobacteria, was inserted into the BioBrick cloning site ³¹. The resulting vector called pAQ1BB provides a convenient tool for transformation of *Synechococcus* sp. PCC 7002 (Figure 2). The target integration site ("landing pad") can be modified by replacing homologous sequences Flank A and Flank B using *Nsi*I/*Eco*RI and *Sal*I/*Sph*I restriction sites respectively. The marker gene for selection can be modified using *Xho*I and *Sal*I restriction sites.



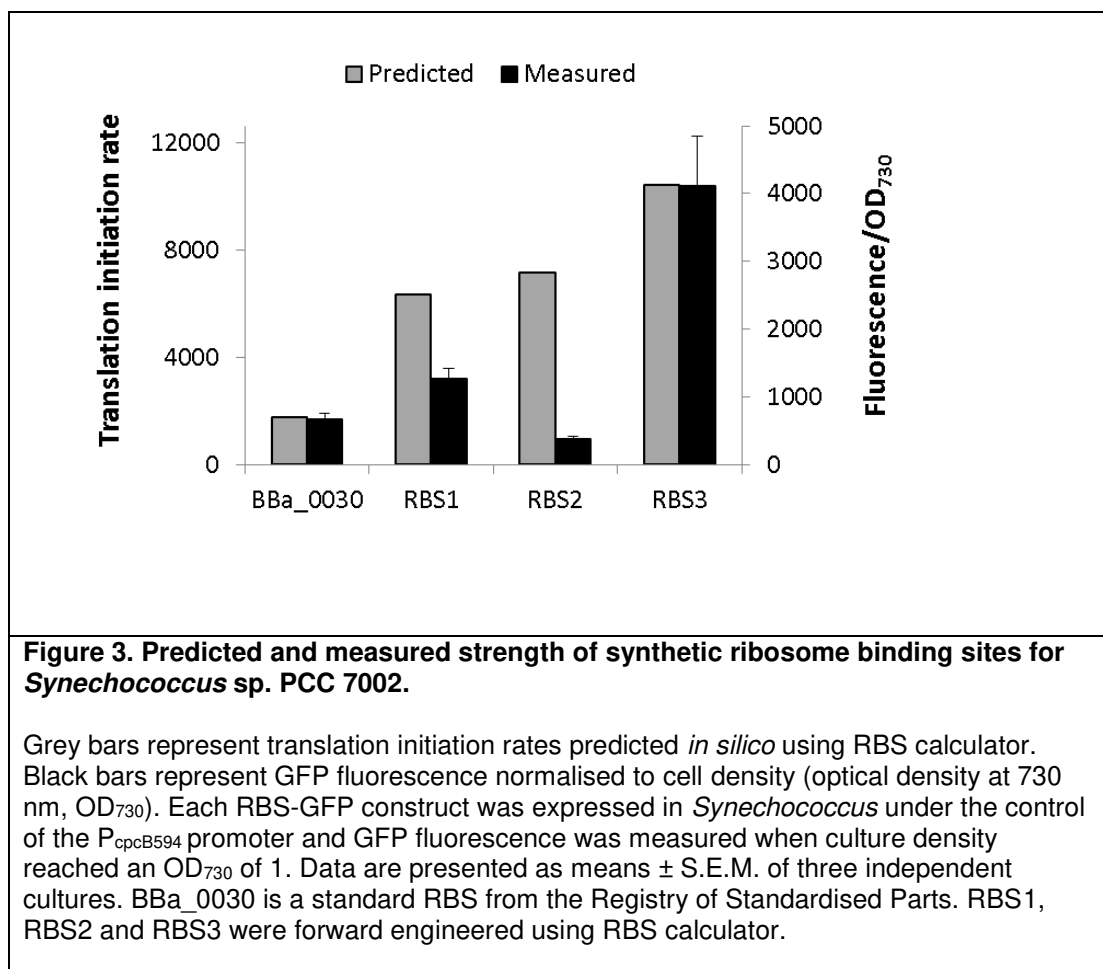
146

147 Using established protocols for natural transformation³⁰, transformation efficiency of
 148 *Synechococcus* with pAQ1BB was initially poor. Increasing both the amount of DNA and
 149 incubation time prior to plating onto selective media significantly enhanced transformation
 150 efficiency (Figure S1). The optimized transformation protocol can be found in the Methods
 151 section.



152

153 Ribosome binding sites (RBSs) play an essential role in translation initiation during protein
 154 synthesis. Software have been developed to design synthetic RBSs considering factors such
 155 as secondary mRNA structure influenced by flanking nucleotide sequences, the Shine-
 156 Dalgarno (SD) sequence complementary to the 16S ribosomal RNA, spacing between the SD
 157 and start codon, and the sequence of the start codon itself ³². We used a web-based RBS
 158 calculator ³³ to design RBS for *Synechococcus* (sequences in Table S2) and assessed the
 159 suitability of three potential RBS for producing green fluorescent protein (GFP). RBS-GFP
 160 were cloned into pAQ1BB and used to transform *Synechococcus*. Similar to a previous report
 161 ²¹ we observed a moderate correlation between predicted and actual translation rates (Figure
 162 3). Both the RBS calculator and the GFP-assay identified RBS3 as the strongest RBS and we
 163 therefore used RBS3 to express *mpusfus*.



164

165 To assess promoter activity in combination with RBS3, we monitored GFP fluorescence in the
 166 P_{cpcB594}:RBS3-GFP expressing cells over the course of culture growth. The experiment
 167 showed that the promoter is most active in the early exponential phase (Figure 4) as
 168 previously suggested ³⁰.

169

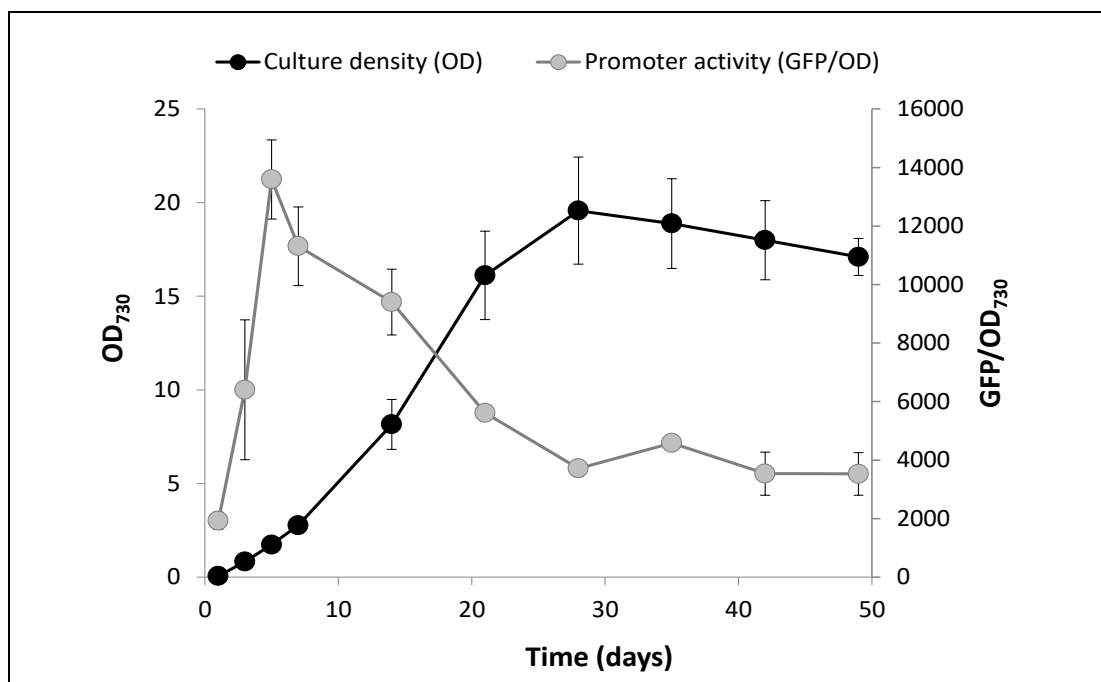


Figure 4. Promoter activity over the course of culture growth

Black points represent culture density (OD₇₃₀). Grey points represent GFP fluorescence normalized to cell density (GFP/OD₇₃₀). RBS3-GFP was expressed in *Synechococcus* under the control of the P_{cpcB594} promoter and GFP fluorescence was measured over the course of culture growth. Data are presented as means ± S.E.M. of three independent cultures.

170

171 ***Mpusfus* is functional in *Synechococcus* sp. PCC 7002**

172 *Synechococcus* was transformed with the plasmid containing P_{cpcB594}: RBS3-*mpusfus*. Initial
 173 growth rates were slightly impaired in the engineered *Synechococcus* and the densities of
 174 stationary phase cultures were slightly lower than in wild type cells (Figure 5A). Mannitol was
 175 measured in the media and in the cell pellet of wild type- and *mpusfus*-expressing cultures.
 176 No mannitol was detected in wild type cultures, confirming a previous report that
 177 *Synechococcus* does not naturally produce mannitol²³. By contrast, mannitol was detected
 178 both in the cellular fraction and in the media of *mpusfus*-expressing cultures, indicating that
 179 the fusion protein is functional in *Synechococcus*. Total mannitol steadily increased over the
 180 course of culture growth to around 100 mg/L in early stationary phase (Figure 5A). Mannitol in
 181 the cellular fraction of the culture increased with culture growth (Figure 5B), but the majority of
 182 mannitol produced was exported into the medium. At the end of the experiment over 99% of
 183 the total mannitol was found in the culture medium.

184 Mannitol production rates were calculated by dividing the amount of mannitol produced
 185 between two time points by time and by mean cell number (OD_{730}) in this time period. As
 186 shown in Figure 5C the production rate increased to $7 \mu\text{g/day/OD}$ on day 28 (mid exponential
 187 phase) and then decreased.

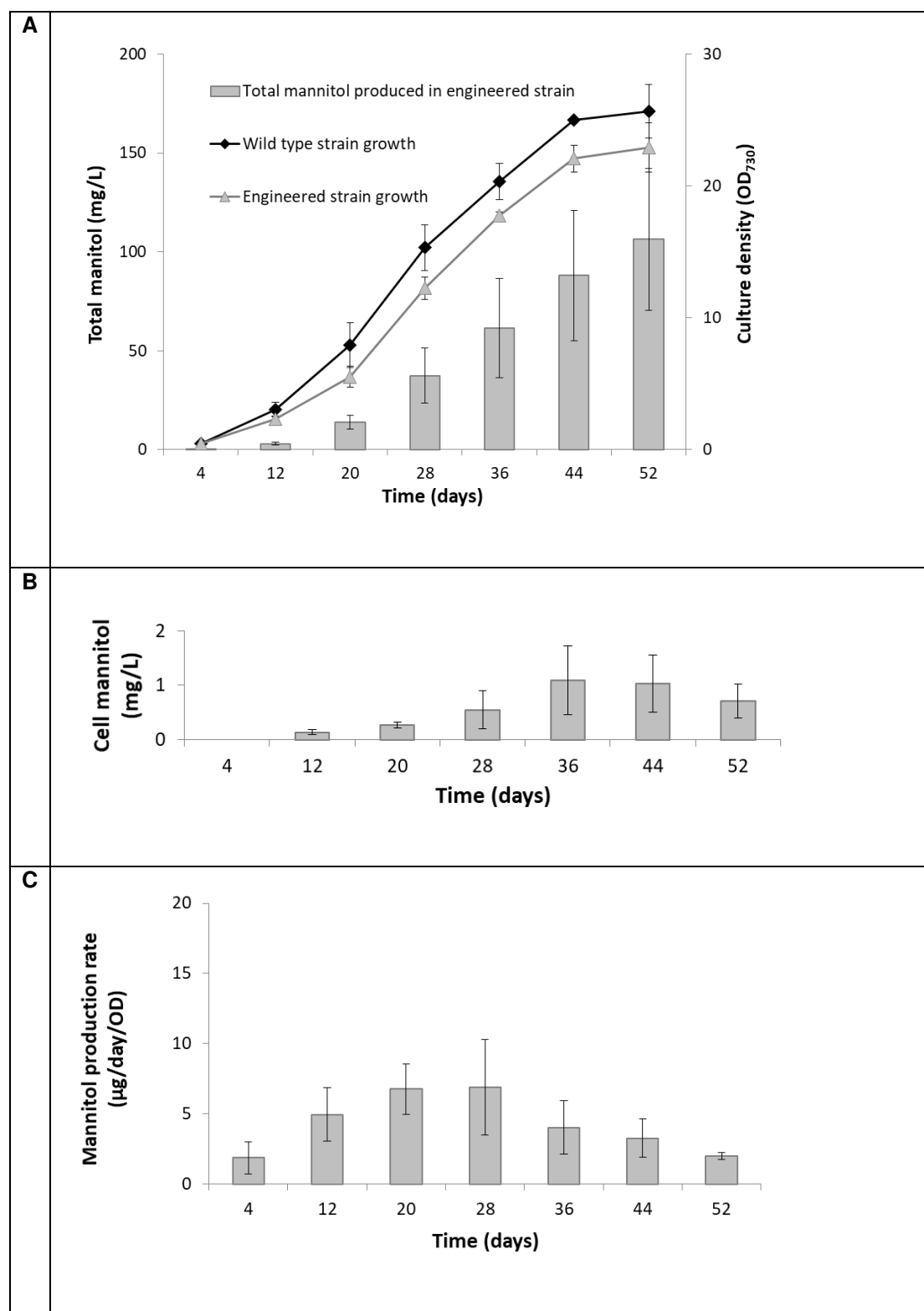


Figure 5. Mannitol production in *mpusfus*-expressing *Synechococcus* cultures.

A: Line graphs represent culture growth as optical density at 730 nm (OD₇₃₀) measured over time in wild type (black symbols) and engineered *Synechococcus* (grey triangles) expressing *mpusfus* under the control of P_{cpcB594} and RBS3. Grey bars represent total mannitol (sum of mannitol measured in the cellular fraction and in the medium).

B: Amount of mannitol (per 1 litre culture) present in the cellular fraction of the engineered *Synechococcus* culture.

C: Mannitol production rate in the engineered *Synechococcus* culture normalised to OD₇₃₀.

All data are means \pm S.E.M. of three independent cultures.

In summary, the results show that expression of a single fusion gene encoding two enzyme functions results in the production of mannitol in heterologous systems that do not naturally synthesize mannitol,

Discussion

Use of fusion genes for biotechnology

Fusion genes encode more than one enzymatic function in one gene. They have potential benefits for biotechnology because they would reduce the number of constructs and transformations required for engineering metabolic pathways. Furthermore, it is hoped that, in a naturally evolved fusion protein, occurrence of adjacent modules catalyzing consecutive steps in a metabolic process will prevent loss of intermediate through efficient substrate channeling. However, functionality of fusion genes/proteins in heterologous systems remained to be proven. In this study, we show that a fusion gene from the green alga *Micromonas pusilla* is functional and sufficient to achieve production of mannitol in prokaryotic systems, namely *E. coli* and *Synechococcus* sp. PCC 7002.

M. pusilla strain CCMP1545 is a unicellular marine green alga that is a potentially valuable source of fusion genes for biotechnology. A high quality draft of its 22 Mb genome is available and resolution continues to improve around the telomeric regions of its 19 chromosomes³⁴⁻³⁵. Bioinformatic analyses have identified a number of fusion genes in the CCMP1545 genome encoding enzymes involved in a variety of cellular processes including pigment production³⁶,

polyamine biosynthesis³⁷, DNA double-strand break repair³⁸ and carbon fixation³⁵. Some *Micromonas* genes have been characterized by heterologous expression to date³⁹⁻⁴², but none of them encode fusion proteins. Therefore, the potential of the *Micromonas* fusion genes for metabolic engineering remained to be explored. The gene employed in this study combined a M1PDH and an M1Pase module and was tested for producing mannitol in prokaryotic model systems.

We first expressed the *mpusfus* fusion gene in *E. coli*. This organism has been previously engineered to develop whole-cell biotransformation systems for mannitol synthesis from glucose⁴³⁻⁴⁴, resulting in mannitol molar yield of 80%⁴³ and 87%⁴⁴. We found that *E. coli* expressing the single *mpusfus* fusion gene produced a single recombinant protein of approximately 100 kDa and produced mannitol, most of which was exported into the medium. This shows that the fusion protein is functional in *E. coli*. However, the achieved mannitol concentration of 218 mg/L under our experimental conditions (molar yield of 2% on glucose) was considerably lower than in the previously engineered strains⁴³⁻⁴⁴. Additional manipulations addressing codon usage, metabolic flux towards substrate, substrate transport and feeding could now be attempted to increase mannitol titres in *E. coli*.

Photosynthetic bacteria provide an opportunity to produce organic compounds from CO₂ without the need of feeding sugars. We were therefore interested to test whether the *mpusfus* fusion gene can be used to produce mannitol in a photo-autotrophic system. *Synechococcus* sp. PCC 7002 is one of the model systems for metabolic engineering of cyanobacteria and has previously been engineered to produce mannitol from F6P using two separate genes (M1PDH from *E. coli* and M1Pase from *Eimeria tenella*)²³. As *E. coli*, *Synechococcus* does not possess an endogenous pathway for mannitol production or breakdown. To facilitate the engineering of *Synechococcus*, we first generated a BioBrick compatible vector and a synthetic ribosomal binding site. The new tools in combination with a previously identified 'super-strong' promoter were confirmed by GFP-assay and are available to the scientific community.

Successful functional expression of *mpusfus* in *Synechococcus* was proven by the appearance of mannitol in the transgenic cultures. As before²³, the majority of mannitol

produced was exported into the media where it can easily be harvested. Since *Synechococcus* does not naturally produce mannitol it is likely that the export occurs through non-specific transport proteins for other compounds. *Synechococcus* and other cyanobacteria have been shown to release low-molecular-weight metabolites when subjected to hypo-osmotic stress ⁴⁵⁻⁴⁶, but the exact transport pathways remain to be identified.

The total amount of mannitol produced in this report is considerably lower than in the previous study ²³, namely around 0.1 g/L compared to 0.6 g/L. Usage of a glycogen-deficient strain helped to increase titres ²³, but the main differences between the two studies lies in the growth rate of the cultures. The highest mannitol-producing strain reported previously ²³ reached a maximal OD₇₃₀ of around 10 within 150 h and achieved the aforementioned mannitol concentration in 300 h. By contrast, our strain grew much more slowly and required 50 days to produce 0.1 g/L mannitol albeit reaching a higher OD₇₃₀ of 25. It is likely that protein turnover over such a long period of time prevents the accumulation and maintenance of substantial amounts of recombinant protein. Protein synthesis then becomes very sensitive to promoter activity, which was maximal during early culture growth (see Fig. 4). A combination of low promoter activity and protein turnover would explain why production rates were very low and decreased even before the cultures entered stationary phase (see Fig. 5C). It can therefore be expected that usage of new promoter(s), which are active during the late stages of growth, and changes in growth conditions, *e.g.* fed-batch cultivation to keep cultures in the production stage for longer, could increase mannitol productivity by engineered *Synechococcus* sp. PCC 7002.

Despite the ability of cyanobacteria to use atmospheric carbon for industrial bioproduction, carbon availability is a key limiting factor in polyol production. The use of fast-growing strains and the development of efficient photo-bioreactors will be crucial to move toward industrial scale production systems that can compete with the traditional sugar-fed cultures. Our demonstration that an algal fusion gene is functional in cyanobacteria presents an important step towards simplifying the generation of recombinant metabolic pathways, and can now be combined with the usual metabolic engineering strategies to overcome metabolic bottlenecks.

265 Metabolic network models have become available for *Synechococcus* ⁴⁷, which should
 266 facilitate this task.

267

268 **Methods**

269 ***Construction of pAQ1BB vector for transformation of *Synechococcus* sp. PCC 7002***

270 The integrative vector pAQ1EX for transformation of *Synechococcus* sp. PCC 7002 ³⁰ was
 271 modified to accommodate BioBrick assembly. Synthetic oligonucleotides were annealed to
 272 generate BioBrick prefix and suffix sequences flanked by restriction site overhangs for
 273 insertion into pAQ1EX using *EcoRI/NcoI* and *NdeI/BamHI* restriction sites respectively (Table
 274 S1). To prevent interference with the BioBrick assembly, an *XbaI* restriction site at the 5' end
 275 of the spectinomycin resistance gene *aadA* was replaced with an *XhoI* restriction site by site-
 276 directed mutagenesis using primers listed in Table S1. Modifications were confirmed by
 277 sequencing and the resulting vector was designated pAQ1BB. To drive transgene expression,
 278 the 594 bp phycocyanin promoter of *Synechocystis* sp. PCC 6803, P_{cpcB594} ³⁰ was amplified
 279 by PCR from genomic DNA using forward primer 5'-
 280 GAATTCGCGGCCGCTTCTAGAGTTCGTTATAAAATAAACTTAACAAATCTATAC-3' and reverse primer 5'-
 281 CTGCAGCGGCCGCTACTAGTAGGAATTAATCTCCTACTTGACTTTATG-3', and inserted into the pAQ1BB
 282 BioBrick cloning site.

283 ***Generation of codon-optimized mpusfus***

284 The 863 aa M1PDH/M1Pase fusion protein of *Micromonas pusilla* strain CCMP1545
 285 (<http://bioinformatics.psb.ugent.be/orcae/annotation/Mipuc/current/Mipuc10g00620>) contains
 286 codons that may reduce translation efficiency in cyanobacteria. Codon optimization for
 287 expression in *Synechococcus* sp. PCC 7002 was therefore performed (GenScript USA Inc.)
 288 using the OptimumGene™ algorithm, which takes into consideration a variety of parameters
 289 important for gene expression efficiency. These include, but are not limited to, codon usage
 290 bias, GC content, mRNA secondary structure, internal ribosome binding sites and restriction

sites. Codon usage bias is scored as a codon adaptation index (CAI), where CAI 1.0 is optimal in the desired expression organism, and CAI > 0.8 is regarded as good in terms of high expression. Following codon optimization, the CAI was improved from 0.66 in the native gene to 0.96 in the codon-optimized gene (Supplementary data file 1, Supplemental Figure 1a). GC content and distribution was also optimized to prolong the half-life of the mRNA, and stem-loop secondary structures were broken to improve mRNA stability and ribosomal binding. Five internal ribosome binding sites were removed to prevent any aberrant translation from within the coding sequence. Finally, six restriction sites involved in BioBrick assembly were removed. The resulting codon-optimized gene was designated *mpusfus*. Details of codon optimization, native and optimized sequences can be found in Supplementary data file 1.

Generation of transgenic Synechococcus sp. PCC 7002 strains

Synthetic ribosome binding sites were designed using the Salis lab RBS calculator³³ and added directly upstream of the transgene during PCR amplification (primer sequences can be found in Table S2). Following sequence confirmation in the pGEM-T® Easy (Promega, UK) vector, the amplified DNA (RBS + gene) was cloned into the pAQ1BB vector, downstream of the P_{cpcB594} promoter. The synthetic expression constructs were integrated into the *Synechococcus* genome by natural transformation. Transformation efficiency was optimised by varying either amount of DNA (1-25 µg) or incubation time (1-3 days) prior to plating on selective media (Figure S1) and the following optimised transformation protocol was used: 1.5 mL culture (OD₇₃₀ 1) was combined with 10 µg circular plasmid DNA and incubated for 72 h under standard growth conditions with minimal sparging. Cells were plated on solid A+ medium with 1.5% w/v agar and 50 µg/ml spectinomycin. Single colonies appeared after 5-7 days. Individual colonies were isolated and grown for characterization. Genomic DNA was isolated using phenol-chloroform extraction⁴⁸, and the correct insertion of the synthetic expression constructs were verified by PCR amplification using primers pAQ1BB-seq-F (5'-CACATGAGAATTTGTCCAG-3') and pAQ1BB-seq-R (5'-CCTTTCGGGCTTGTTAG-3') and sequencing.

319 ***Synechococcus* sp. PCC 7002 growth**

320 *Synechococcus* sp. PCC 7002 cultures were grown in A+ medium¹⁸ (containing 300 mM
 321 NaCl) at 30 °C with photoperiod 16 h/8 h light/dark, light intensity 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$
 322 and sparged with humidified ambient air (standard conditions). Cultures of transformed
 323 *Synechococcus* were supplemented with 25 $\mu\text{g/ml}$ spectinomycin. For mannitol analyses,
 324 cultures were grown to OD₇₃₀ 1-3, diluted to OD₇₃₀ 1 and 1.3 ml was inoculated to 400 ml A+
 325 medium. At regular intervals throughout culture growth, OD₇₃₀ was measured and 20 ml of
 326 culture was harvested by centrifugation at 4,000 *g* for 15 min. Supernatant were transferred to
 327 fresh tubes and stored at -20 °C. Cells were resuspended in 3 ml fresh Medium A+,
 328 transferred to a fresh 15 ml Falcon tube, centrifuged at 4,000 *g* for 15 min at 4 °C,
 329 supernatants were discarded and pellets were frozen at -20 °C. For subsequent analysis,
 330 cells were freeze-dried overnight at -50 °C and 0.13 millibar, and re-suspended in 1 ml of
 331 Tris-HCl 10 mM pH 8.0. After five freeze-thaw cycles, cell suspensions were sonicated (5 x
 332 30 sec) using an ultrasonic processor VC50 sonicator (Jencons) and cell debris pelleted by
 333 centrifugation 15 min at 12,500 *g*. Aliquots of the cell lysates were used for determination of
 334 intracellular mannitol concentration.

335 **Generation of transgenic *E. coli* strain**

336 *Mpusfus* DNA without the initial start codon was amplified from the plasmid carrying the
 337 codon-optimized gene with the forward primer
 338 5'-GGGGGGGGATCCAATAGTGCCGTGACCATCAGCC-3' (*Bam*HI restriction site underlined) and the
 339 reverse primer 5'-CCCCCGAAATTCTTAGCGGGGATTGGGATCTTC-3' (*Eco*RI restriction site
 340 underlined). The PCR fragment obtained was cloned into the vector pFO4 as previously
 341 described⁴⁹, producing the plasmid pMPUSFUS and allowing the recombinant proteins to be
 342 fused to a six-histidine tag at their N-terminus. This plasmid was subsequently transformed in
 343 *E. coli* expression strain BL21 (DE3). For gene induction and functional assays, three distinct
 344 recombinant clones were pre-cultured in 5 ml of M9 medium supplemented with 10 g/L of
 345 glucose and 0.1 g/L ampicillin overnight at 37 °C and 200 rpm. The following day, new
 346 cultures were started at OD₆₀₀ 0.1 under the same conditions. When OD₆₀₀ 0.5 was attained,
 347 cultures were divided into two tubes, and isopropyl β -D-1-thiogalactopyranoside (IPTG) was

added in one of them at a final concentration of 1 mM. Incubation was carried out for 20 h at 25 °C and 200 rpm, and cultures reached a final OD₆₀₀ of 2.5-3.0 in both induced and non-induced conditions. Samples were then harvested by centrifugation at 3,500 *g* for 10 min. Supernatants and cell pellets were frozen individually at -20 °C for downstream analyses. To determine intracellular mannitol concentration, cells were re-suspended in 600 µl of buffer Tris-HCl 25 mM pH 7.5 and NaCl 200 mM, and disrupted by four pulses of 15 sec of sonication using an ultrasonic processor VC50 sonicator (Jencons). Cell debris were pelleted by centrifugation at 14,000 *g* and 4 °C for 20 min; supernatants were used for mannitol analysis and considered as soluble fractions for protein gel and Western-blot analysis. Proteins were quantified using the Pierce™ Coomassie Plus Assay Kit (ThermoFischer Scientific) according to manufacturer's instructions. Three µg of proteins of each fractions were loaded on a 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Biorad). After separation, proteins were transferred to nitrocellulose membrane using the iBlot 2 Dry blotting System (ThermoFischer Scientific). Recombinant proteins were visualized with a conjugated monoclonal anti-polyhistidine-peroxidase antibody (Sigma-Aldrich) detected by the SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFischer Scientific).

Measurement of GFP

RBS and promoter activity was assessed in transgenic cyanobacteria using a GFP reporter encoded by BioBrick part BBa_E0040. Cultures were harvested for GFP measurements at the same point during the diurnal cycle and, when appropriate, growth stage. Optical density was adjusted to OD₇₃₀ 0.25-0.30 in fresh A+ media and fluorescence was measured using a LS 55 Luminescence Spectrophotometer (PerkinElmer, UK) using 480 nm excitation and 514 nm emission wavelengths.

Mannitol analysis

To determine mannitol production, 50-200 µl aliquots of culture media or bacterial extracts were analyzed with the D-Mannitol/L-Arabitol assay kit (K-MANOL, Megazyme) according to the manufacturer's instructions.

Associated content

Supporting information

Details for codon optimization of *Micromonas pusilla* M1PDH/M1Pase fusion gene (PDF)

List of primers used for pAQ1BB construction and to generate RBS+gene constructs (XLSX)

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Author contributions

MAM, AA, and TT conceived the study. Experimental data were generated by MAM, SS, and

TT. All authors analyzed data. MAM, AA, and TT wrote the manuscript.

Notes

The authors declare no competing financial interest.

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References

1. Singh, R., et al., Parihar, P., Singh, M., Bajguz, A., Kumar, J., Singh, S., Singh, V.P., and Prasad, S.M. (2017) Uncovering potential applications of cyanobacteria and algal metabolites in biology, agriculture and medicine: Current status and future prospects. *Front. Microbiol.* **8**, 515.
2. Gustavsson, M., and Lee, S.Y. (2016) Prospects of microbial cell factories developed through systems metabolic engineering. *Microb. Biotechnol.* **9**, 610-617.
3. Jeschek, M., Gerngross, D., and Panke, S. (2017) Combinatorial pathway optimization for streamlined metabolic engineering. *Curr. Opin. Biotechnol.* **47**, 142-151.
4. Chae, T.U., Choi, S.Y., Kim, J.W., Ko, Y.S., and Lee, S.Y. (2017) Recent advances in systems metabolic engineering tools and strategies. *Curr. Opin. Biotechnol.* **47**, 67-82.
5. Nielsen, J., Archer, J., Essack, M., Bajic, V.B., Gojobori, T., and Mijakovic, I. (2017) Building a bio-based industry in the Middle East through harnessing the potential of the Red Sea biodiversity. *Appl. Environ. Microbiol.* **101**, 4837-4851.
6. Jensen, M.K., and Keasling, J.D. (2015) Recent applications of synthetic biology tools for yeast metabolic engineering. *FEMS Yeast Res.* **15**, 1-10.
7. Miles, E.W., Rhee, S., and Davies, D.R. (1999) The molecular basis of substrate channeling. *J. Biol. Chem.* **274**, 12193-12196.
8. Dai, Y., Meng, Q., Mu, W., and Zhang, T. (2017) Recent advances in the applications and biotechnological production of mannitol. *J. Funct. Foods* **36**, 404-409.
9. Oddo, E., Saiano, F., Alonzo, G., Bellini, E. (2002) An investigation of the seasonal pattern of mannitol content in deciduous and evergreen species of the oleaceae growing in northern Sicily. *Ann. Bot.* **90**, 239-243.
10. Saha, B.C., and Racine, F.M. (2011) Biotechnological production of mannitol and its applications. *Appl. Microbiol. Biotechnol.* **89**, 879-891.
11. Hays, S.G., and Ducat, D.C. (2015) Engineering cyanobacteria as photosynthetic feedstock factories. *Photosynth. Res.* **123**, 285-295.
12. Hagemann, M. and Hess, W.R., (2018) Systems and synthetic biology for the biotechnological application of cyanobacteria. *Curr. Opin. Biotechnol.* **49**, 94-99.

- 430 13. Cassier-Chauvat, C., Dive, V., and Chauvat, F. (2017) Cyanobacteria: photosynthetic
 431 factories combining biodiversity, radiation resistance, and genetics to facilitate drug discovery.
 432 *Appl. Environ. Microbiol.* 101, 1359-1364.
- 433 14. Al-Haj, L., Lui, Y.T., Abed, R.M., Gomaa, M.A., and Purton, S. (2016) Cyanobacteria as
 434 chassis for industrial biotechnology: Progress and prospects. *Life* 6, E42.
- 435 15. Minas, K., Karunakaran, E., Bond, T., Gandy C., Honsbein, A., Madsen, M., Amezaga, J.,
 436 Amtmann, A., Templeton, M.R., Biggs, C.A., and Lawton, L. (2015) Biodesalination: an
 437 emerging technology for targeted removal of Na⁺ and Cl⁻ from seawater by cyanobacteria.
 438 *Desalin. Water Treat.* 55, 2647-2668.
- 439 16. Nomura, C.T., Sakamoto, T., and Bryant, D.A. (2006) Roles for heme-copper oxidases in
 440 extreme high-light and oxidative stress response in the cyanobacterium *Synechococcus* sp.
 441 PCC 7002. *Arch. Microbiol.* 185, 471-479.
- 442 17. Van Baalen, C. (1962) Studies on marine blue-green algae. *Bot. Mar.* 4, 129-139.
- 443 18. Stevens, S.E., and Porter, R.D. (1980) Transformation in *Agmenellum quadruplicatum*.
 444 *Proc. Natl. Acad. Sci. U. S. A.* 77, 6052-6056.
- 445 19. Frigaard, N.U., Sakuragi, Y., and Bryant, D.A. (2004) Gene inactivation in the
 446 cyanobacterium *Synechococcus* sp. PCC 7002 and the green sulfur bacterium *Chlorobium*
 447 *tepidum* using in vitro-made DNA constructs and natural transformation. *Methods Mol. Biol.*
 448 274, 325-340.
- 449 20. Ruffing, A.M., Jensen, T.J., and Strickland, L.M. (2016) Genetic tools for advancement of
 450 *Synechococcus* sp. PCC 7002 as a cyanobacterial chassis. *Microb. Cell Fact.* 15, 190.
- 451 21. Markley, A.L., Begemann, M.B., Clarke, R.E., Gordon, G.C., and Pfleger, B.F. (2015)
 452 Synthetic biology toolbox for controlling gene expression in the cyanobacterium
 453 *Synechococcus* sp. strain PCC 7002. *ACS Synth. Biol.* 4, 595-603.
- 454 22. Zess, E.K., Begemann, M.B., and Pfleger, B.F. (2016) Construction of new synthetic
 455 biology tools for the control of gene expression in the cyanobacterium *Synechococcus* sp.
 456 strain PCC 7002. *Biotechnol. Bioeng.* 113, 424-432.
- 457 23. Jacobsen, J.H., and Frigaard N.U. (2014) Engineering of photosynthetic mannitol
 458 biosynthesis from CO₂ in a cyanobacterium. *Metab. Eng.* 21, 60-70.

- 459 24. Michel, G., Tonon, T., Scornet, D., Cock, J.M., and Kloareg, B. (2010) Central and storage
460 carbon metabolism of the brown alga *Ectocarpus siliculosus*: insights into the origin and
461 evolution of storage carbohydrates in Eukaryotes. *New Phytol.* 188, 67-81.
- 462 25. Bonin, P., Groisillier, A., Raimbault, A., Guibert, A., Boyen, C., and Tonon T. (2015)
463 Molecular and biochemical characterization of mannitol-1-phosphate dehydrogenase from the
464 model brown alga *Ectocarpus* sp. *Phytochemistry* 117, 509-20.
- 465 26. Groisillier, A., Shao, Z., Michel, G., Goulitquer, S., Bonin, P., Krahulec, S., Nidetzky, B.,
466 Duan, D., Boyen, C., and Tonon, T. (2014) Mannitol metabolism in brown algae involves a
467 new phosphatase family. *J. Exp. Bot.* 65, 559-570.
- 468 27. Rousvoal, S., Groisillier, A., Dittami, S.M., Michel, G., Boyen, C., and Tonon, T. (2011)
469 Mannitol-1-phosphate dehydrogenase activity in *Ectocarpus siliculosus*, a key role for
470 mannitol synthesis in brown algae. *Planta* 233, 261-273.
- 471 28. Tonon, T., Li, Y., and McQueen-Mason, S. (2017) Mannitol biosynthesis in algae: more
472 widespread and diverse than previously thought. *New Phytol.* 213, 1573-1579.
- 473 29. Shetty, R.P., Endy, D., and Knight, T.F.J. (2008) Engineering BioBrick vectors from
474 BioBrick parts. *J. Biol. Eng.* 2, 5.
- 475 30. Xu, Y., Alvey, R.M., Byrne, P.O., Graham, J.E., Shen, G., and Bryant, D.A. (2011)
476 Expression of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for
477 high-level gene expression in *Synechococcus* sp. PCC 7002. *Methods Mol. Biol.* 684, 273-
478 293.
- 479 31. Zhou, J., Zhang, H., Meng, H., Zhu, Y., Bao, G., Zhang, Y., Li, Y., and Ma, Y. (2014)
480 Discovery of a super-strong promoter enables efficient production of heterologous proteins in
481 cyanobacteria. *Sci. Rep.* 4, 4500.
- 482 32. Reeve, B., Hargest, T., Gilbert, C., and Ellis, T. (2014) Predicting translation initiation
483 rates for designing synthetic biology. *Front. Bioeng. Biotechnol.* 2, 1.
- 484 33. Salis, H.M., Mirsky, E.A., and Voigt, C.A. (2009) Automated design of synthetic ribosome
485 binding sites to control protein expression. *Nat. Biotechnol.* 27, 946-950.
- 486 34. van Baren. M.J., Bachy, C., Reistetter, E.N., Purvine, S.O., Grimwood, J., Sudek, S., Yu,
487 H., Poirier, C., Deerinck, T.J., Kuo, A., et al. (2016) Evidence-based green algal genomics
488 reveals marine diversity and ancestral characteristics of land plants. *BMC Genomics* 17, 267.

- 489 35. Worden, A.Z., Lee, J.H., Mock, T., Rouzé, P., Simmons, M.P., Aerts, A.L., Allen, A.E.,
 490 Cuvelier, M.L., Derelle, E., Everett, M.V., et al. (2009) Green evolution and dynamic
 491 adaptations revealed by genomes of the marine picoeukaryotes *Micromonas*. *Science* 324,
 492 268-272.
- 493 36. Blatt, A., Bauch, M.E., Pörschke, Y., and Lohr, M. (2015) A lycopene β -cyclase/lycopene
 494 ϵ -cyclase/light-harvesting complex-fusion protein from the green alga *Ostreococcus*
 495 *lucimarinus* can be modified to produce α -carotene and β -carotene at different ratios. *Plant J.*
 496 82, 582-595.
- 497 37. Green, R., Hanfrey, C.C., Elliott, K.A., McCloskey, D.E., Wang, X., Kanugula, S., Pegg,
 498 A.E., and Michael, A.J. (2011) Independent evolutionary origins of functional polyamine
 499 biosynthetic enzyme fusions catalyzing de novo diamine to triamine formation. *Mol. Microbiol.*
 500 81, 1109-1124.
- 501 38. Yoshida, T., Claverie, J.-M., and Ogata, H. (2011) Mimivirus reveals Mre11/Rad50 fusion
 502 proteins with a sporadic distribution in eukaryotes, bacteria, viruses and plasmids. *Viol. J.* 8,
 503 427-437.
- 504 39. Shi, H., Chen, H., Gu, Z., Zhang, H., Chen, W., and Chen, Y.Q. (2016) Application of a
 505 delta-6 desaturase with α -linolenic acid preference on eicosapentaenoic acid production in
 506 *Mortierella alpina*. *Microb. Cell Fact.* 15, 117.
- 507 40. Duanmu, D., Bachy, C., Sudek, S., Wong, C.H., Jiménez, V., Rockwell, N.C., Martin, S.S.,
 508 Ngan, C.Y., Reistetter, E.N., van Baren M.J., et al. (2014) Marine algae and land plants
 509 share conserved phytochrome signaling systems. *Proc. Natl. Acad. Sci. U. S. A.* 111, 15827-
 510 15832.
- 511 41. Petrie, J.R., Shrestha, P., Mansour, M.P., Nichols, P.D., Liu, Q., and Singh, S.P. (2010)
 512 Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids into plants using an
 513 acyl-CoA $\Delta 6$ -desaturase with $\omega 3$ -preference from the marine microalga *Micromonas pusilla*.
 514 *Metab. Eng.* 12, 233-240.
- 515 42. Lada, A.G., Krick, C.F., Kozmin, S.G., Mayorov, V.I., Karpova, T.S., Rogozin, I.B., and
 516 Pavlov, Y.I. (2011) Mutator effects and mutation signatures of editing deaminases produced
 517 in bacteria and yeast. *Biochemistry (Moscow)* 76, 131-146.

- 518 43. Kaup, B., Bringer-Meyer, S., and Sahm, H. (2005) D-mannitol formation from D-glucose in
519 a whole-cell biotransformation with recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.*
520 69, 397-403.
- 521 44. Reshamwala, S. M., Pagar, S. K., Velhal, V. S., Maranholakar, V. M., Talangkar, V. G.,
522 and Lali, A. M. (2014) Construction of an efficient *Escherichia coli* whole-cell biocatalyst for D-
523 mannitol production. *J. Biosci. Bioeng.* 118, 628-631.
- 524 45. Hagemann, M. (2011) Molecular biology of cyanobacterial salt acclimation. *FEMS*
525 *Microbiol. Rev.* 35, 87-123.
- 526 46. Xu, Y., Guerra, L. T., Li, Z., Ludwig, M., Dismukes, G. C., and Bryant, D. A. (2013) Altered
527 carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC
528 7002: Cell factories for soluble sugars. *Metab. Eng.* 16, 56-67.
- 529 47. Gudmundsson, S., Nogales, J. (2015) Cyanobacteria as photosynthetic biocatalysts: a
530 systems biology perspective. *Mol. BioSyst.* 11, 60-70.
- 531 48. Tamagnini, P., Troshina, O., Oxelfelt, F., Salema, R., and Lindblad, P. (1997)
532 Hydrogenases in *Nostoc* sp. strain PCC 73102, a strain lacking a bidirectional enzyme. *Appl.*
533 *Environ. Microbiol.* 63, 1801-1807.
- 534 49. Groisillier, A., Hervé, C., Jeudy, A., Rebuffet, E., Pluchon, P.F., Chevolot, Y., Flament, D.,
535 Geslin, C., Morgado, I.M., Power, D., et al. (2010) MARINE-EXPRESS: taking advantage of
536 high throughput cloning and expression strategies for the post-genomic analysis of marine
537 organisms. *Microb. Cell Fact.* 9, 45.

538